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TECHNICAL REPORT

The Impact of Hazardous Chemicals On Macrophages

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14. ABSTRACT
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Technical Report

Introduction

The goals of this study were to identify the impact of hazardous chemicals on macrophages, determine how the macrophages respond these chemicals, and use these signatures to develop novel detection technologies. As a model for this system we selected toxic industrial chemicals and used mouse macrophages as the target cell type.

Although less toxic than military chemical warfare agents, toxic industrial chemicals (TICs) have hazardous properties and are substantially easier to obtain than military class agents (7). Literally thousands of chemicals routinely are used in manufacturing, agriculture, water treatment, and many other applications. Under certain exposure scenarios (e.g., dose, exposure pathway, target receptor), many of those chemicals exhibit toxicity and exposure can cause mortality or morbidity. As a result, exposures to TICs primarily via the inhalation and dermal absorption pathways are directly associated with a variety of human diseases including cancer, cardiovascular disease, and respiratory illnesses. Those exposures are also known to interrupt immune responses (5). And, based on previous studies, it is apparent that toxic chemicals can cause inflammation and also suppress immune responses (1), both of which could have severe consequences to the health of individuals. Inflammation triggered by TICs can cause localized tissue damage, which can be substantial if the TIC induces inflammation in the upper and lower respiratory tract (6). In a like manner, the immunosuppressive activities of TICs increase human susceptibility to infection and reduce the host's ability to fight malignancies (9).

Interestingly enough, despite these established associations between TICs and lethal or sub-lethal responses, knowledge about the actual cellular processes interrupted by these chemicals, or how these chemicals alter an otherwise normal immune response by monocytic cells such as macrophages is limited. Macrophages are of particular interest because these cells can potentiate or downregulate immune responses, they are essential components of the host's innate immune response, and are among the first cells to encounter an invading pathogen or come in contact with molecules that are non-self (2). Following this early step in immunity, macrophages intensify the immune response by signaling to and directly activating other host immune cells. Macrophages can be inactivated by a variety of natural mechanisms that include the production of anti-inflammatory cytokines and down regulation of mitogen activated protein kinases (2). Processes such as these prevent the macrophage from over stimulating the host immune system, which would otherwise lead to shock and death. However, these regulatory mechanisms also make the macrophage susceptible to inactivation by sources outside of the normal host immune responses. Indeed, many bacterial pathogens are successful in causing disease because the organisms have evolved factors that down regulate macrophages (3). Thus, inactivation of macrophages may represent a crucial aspect of inflammation or immunosuppression mediated by TICs.

Previous studies reveal that toxic chemicals can also suppress macrophage function or promote macrophage death by triggering apoptotic pathways (1, 4). For

example, diesel exhaust particles (DEP) are known to generate reactive oxygen intermediates that trigger oxidative stress and caspase activation in macrophages (4). Among the chemicals in DEP are halogenated hydrocarbons and polycyclic aromatic hydrocarbons, which have also been associated with macrophage cell death and the activation of stress-related pathways in macrophages. Indeed, work by Ng et al. revealed that AP-1 signaling is activated in macrophages following exposure to chemicals found in DEP (8). Yet whether this process is triggered by other TICs has not been determined.

In the current study we examined the effects of sublytic doses of selected TICs on macrophage function, with a particular emphasis on identifying changes in expression of cytokines and chemokines. These altered patterns in immune function were linked to changes in the activation state of intracellular signaling pathways. Overall the findings reveal that sublytic levels of TICs can alter the profile of cytokines and chemokines expressed by macrophages, with a pattern that suggests immunosuppression. Finally, we demonstrate that critical changes in immune function correlate with activation of kinase signaling pathways in macrophages. These data provide insight into the mechanism by which TICs can invoke immunomodulatory effects on individuals exposed to these chemicals. And, if these response evoke unique 'signatures' with respect to cytokine and chemokine production, this may support development of a cell-based system of biomarkers for identifying and differentiating chemical-specific exposures. Finally, we use this information to design macrophages to fluoresce in the presence of TICs.

MATERIALS & METHODS

Chemicals. All selected toxic industrial chemicals (TICs) were of low toxicity and were obtained from Sigma-Aldrich Chemical (St. Louis, MO). The lists of the TICs with their abbreviations are described in Table 1. A brief description of a few of the TICs is as follows:

Allyl isothiocyanate (AITC): a clear to pale yellow synthetic mustard oil with low water solubility. It has a wide range of applications including manufacturing industrial chemicals such as in freezing solutions, fabric dyeing, printing, photographing industry, electroplating, pharmaceuticals, pesticides etc. Naturally occurring AITC is known to have some anti-tumor & anti-oxidant properties by inducing the activity of phase II detoxification enzymes in the urinary bladder.

Arsenic (III) chloride [Ars(III)Cl]: Arsenic is one of the highly poisonous metallic elements derived from the natural environment. It causes lung tumors by inhalation, and cancers of the skin and bladder by ingestion. Arsenic compounds are used as agricultural pesticides (such as copper and lead arsenates), wood preservative, for glass making, in the production of semiconductor devices like integrated circuits and also used in laser and light-emitting diodes (LEDs).

Butyl chloroformate (ButClF) & *Isopropyl chloroformate* (IsoPropClF): They are moisture sensitive clear liquid with sharp odor, insoluble in water and stable under ordinary conditions. ButClF & IsoPropClF are used as an intermediate for the production of peroxide chemicals, dyes, crop protection chemicals, pharmaceuticals

and agrochemical industry. *Isobutyl chloroformate* (IsoButClF) is a peptide coupling agent and derivatizing reagent for GC analysis of amino acids and amines.

Carbon tetrachloride (CCl4): a colorless, dense, highly poisonous, highly volatile and nonflammable liquid with a characteristic aromatic odor, belonging to the family of organic halogen compounds. It is a known ozone layer depleting agent with a half-life of more than 30 years. It was used in petroleum refining, pharmaceutical manufacturing, as fire extinguishers in mixtures with potent fumigants to reduce the fire hazard, in manufacture of Freon refrigerants and propellants for aerosol cans.

Chloroacetyl chloride (ClACl): a clear to slightly yellowish fuming liquid with a strong pungent odor similar to that of hydrochloric acid. It is used as agrochemicals, pharmaceuticals, dyes, textile auxiliaries, paper modifiers, plastic additives, and peroxide compounds.

Hydriodic acid (HA): It is the aqueous pale yellow solution of gas hydrogen iodide that decolorizes after exposure to light. It is a strong acid and reducing agent used as raw materials for pharmaceuticals, analytical reagent as well as in organic synthesis and making iodine salts. *4,4'-Methylenebis* (4'4'MetBis): also known as phenyl isocyanate, its uses include UV curable and latent catalyst-containing polymerization compositions. All of the TICs were used at different dilution factors rather than molar concentration in 10% FBS containing DMEM such as for cytotoxic dose the ratio was 1:1,000 and for chemokine-cytokine production and signaling pathway 1:10,000. No significant changes of the chemicals were observed in 10% FBS containing DMEM.

Cell culture. The RAW 264.7 mouse macrophage-like cell line derived from ascites of BALB/c mice were obtained from the American Type Culture Collection (ATCC, TIB-71, Manassas, VA). These cells were maintained in tissue culture-grade T-75 flasks in the presence of Dulbecco's Modified Essential Medium (DMEM, Gibco) supplemented with 10% defined Fetal Bovine Serum (FBS, ATCC). Cells were subcultured every 2-3 days at 37°C in a humidified incubator containing 5.0% CO_2 when they achieve 70-80% confluence. Prior to TIC treatment, RAW 264.7 cells were seeded at $1x10^4$ cells/well (96 well plate) and $7.5x10^4$ cells/well (24 well plate) in 500µl of DMEM with 10% FBS for cytotoxicity and multiplexing assays respectively. After 15 h, cells were treated with relevant chemicals, pre-added in the 10% FBS containing DMEM at a ratio of 1:1,000 and 1:10,000 for % LDH release and multiplex chemokine-cytokine/signaling pathway experiments respectively. In all experiments, RAW 264.7 cells were passed fewer than 10 times prior to infection.

Assessment of TIC treated macrophage death. Macrophage viability in cultures after TIC treatment were assessed by trypan blue staining and by a lactate dehydrogenase (LDH) release assay using the CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega, Madison, WI) following the instructions of the manufacturers. RAW 264.7 cells were seeded in 96-well plates at $1x10^4$ cells/well in 500µl of culture medium and grown at 37°C for 15 h. Cells were washed once with the same culture medium before treatment with 1,000th dilution of different TICs for 8-12 h and culture supernatants were harvested for analysis of LDH release. After a 10-minute incubation with the substrate mix, the reaction was stopped and

microplate was read on Fluostar OPTIMA plate reader (BMG Lab Technologies, Offenburg, Germany) at 544 nm excitation and 590 nm emission for LDH quantification. Total percentage of LDH released expressed as cytotoxicity was measured against 100% lysis of untreated macrophages using Triton X-100 (100 µl/well of a 9% solution) as lysis solution included in the CytoTox-ONE kit. Cells cultured in normal culture medium without TIC components were used as negative control. Cytotoxicity values were calculated as follows: % cytotoxicity = 100 × [(fluorescence reading of sample well) -(blank)]/[(fluorescence reading of 100% lysed cells) -(blank)].

Determination of chemokine concentration in TIC-treated macrophage. For stimulation, we transferred $7.5x10^4$ cells/well to a 24-well polystyrene culture plate in DMEM with 10% FBS & respective toxic chemicals. After 5 h incubation at 37°C in a humidified incubator containing 5.0% CO_2, we harvested the culture medium for chemokine/cytokine measurement. 500 ng/ml of LPS was included as positive control and normal RAW 264.7 cell culture in 10% FBS containing DMEM without TIC components was used as negative control. We used commercially available kits for measuring multiple chemokines, obtained from Millipore (Milliplex Map Kit, Bedford, MA, USA) in accordance with the manufacturer's recommendations. Luminex 100 system (xMAP) based multiplex analysis allows measurements of a large number of analytes simultaneously in each sample. Pro-inflammatory immunomodulating chemokine levels, including MCP-1, MIP-1α, MIP-1β, MIP-2, and RANTES were determined in supernatants from different TICs stimulated RAW

264.7 cells by Mouse Cytokine/Chemokine kit using fluorescently labeled microsphere beads and a Luminex reader. Briefly, 25µl of TIC stimulated RAW 264.7 culture supernatant were incubated with 25µl of fluorescently labeled microsphere bead at 4°C for overnight. Next, the 96 well microplate was washed once and incubated with 25µl of detection antibody for 1 h at room temperature. Finally, after 30 min of incubation with 25µl of streptavidin-phycoerythrin and one time wash the plate was run on the Luminex platform. The concentrations of different chemokines were calculated by comparison with standard curves determined with the known concentration of chemokines provided by the manufacturer.

Cytokine measurement in toxic chemical exposed RAW 264.7. Different toxic chemical treated macrophage culture supernatants were analyzed with a multiplex assay (Milliplex Map Kit, Bedford, MA, USA) for simultaneous quantitation of 9 cytokines: IL-1α, IL-1β, IL-6, IL-10, IL-12p70, TNFα, IFNγ, G-CSF & GM-CSF. The same TIC stimulated culture medium as mentioned earlier was used for cytokine detection and the same protocol was also followed for Luminex plate assay.

Intracellular signaling pathway induction by TIC-exposed macrophage. Multiplex quantification of phosphoprotein concentrations in chemically treated cell lysate was performed on the Luminex platform (Luminex, http://www.luminexcorp.com). Levels of phosphoprotein to detect the changes in phosphorylated Erk/MAP kinase 1/2, STAT3, JNK, p70 S6 kinase, IκB-α, STAT5A/B,

CREB and p38 were determined using the Beadlyte 8-plex Multi-Pathway Signaling kit by Upstate (Charlottesville, VA, http://www.upstate.com) according to the manufacturer's instructions. Beadlyte Cell Signaling Universal Lysis buffers (containing proprietary formulation of Tris-buffered salts, phosphatase inhibitors including 1mM sodium orthovanadate) and Cell Signaling Universal Assay buffers (containing Tris-buffered salts, animal protein, 0.05% azide) were obtained from Upstate, USA. Briefly, 5 h after treatment with different TICs, medium was removed and cells were lysed in 200 µl of Beadlyte lysis buffer. Protein concentration was determined by Bio-Rad D_C Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) and 25µg of total protein/well was used for the signaling assay. Data analysis was performed using the Bio-Plex Manager software (Bio-Rad Laboratories, http://www.bio-rad.com).

Immunoblot analysis of p38 activation by different TICs. The same RAW 264.7 cell lysates of the signaling assay as mentioned earlier were used for immunoblot analysis of the p38 activation to confirm the consistency of Beadlyte signaling data. Cellular proteins (5 µg) were separated by 12% SDS-PAGE and subsequently transferred to a polyvinylidine difluoride membrane for Western blotting. Membranes were incubated with rabbit polyclonal p38 & phospho-p38 specific primary antibody (Cell Signaling), followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Reacting proteins were detected using the enhanced chemiluminescence detection system (Amersham Pharmacia) and visualized via autoradiography.

Statistical analysis. Data were expressed as means ± SEM of observations (n=3-5). Statistical analysis among experimental groups was performed using a two-tailed Student's t test (GraphPad Prism 4 Software, http://www.graphpad.com). Probability values p < 0:05 were considered as a significant. All sample collections and experiments were performed in triplicates or duplicates under the same condition and repeated at least two times.

Production of luciferase gene reporters in RAW 264.7 cells. RAW 264.7 cells were transduced with lentivirus particles containing luciferase gene reporters purchased from SABioscience. These gene reporters possess a minimal promoter plus enhancer elements specific for each transcription factor response element examined in these studies (RAW-AP1, RAW-C/EBP, RAW-CRE, RAW-GAS, RAW-ISRE, RAW-NFAT, RAW-NF-κB, RAW-RBP-Jκ, RAW-SMAD, RAW-SRE, RAW-STAT3, and RAW-TCF). Also, a gene reporter that contained only the minimal promoter was used as a control (RAW-NEG). RAW 264.7 cells were infected with lentiviral particles at a m.o.i. of 10 in the presence of 8 μg/ml polybrene. The virus was incubated with the cells for 24 h at 32°C, and then selection for stably transduced cells was performed with 3.5 μg/ml of puromycin. The stably transduced cells were subsequently maintained with 3.5 μg/ml puromycin until 24 h before experiments.

Luciferase Assay. For each condition, luciferase expression in RAW-AP1, RAW-C/EBP, RAW-CRE, RAW-GAS, RAW-ISRE, RAW-NFAT, RAW-NF-κB, RAW-RBP-Jκ,

RAW-SMAD, RAW-SRE, RAW-STAT3, RAW-TCF, or RAW-Neg was determined using the Luciferase Assay System (Promega) followed by measurement of luminescence signal with a Victor3 (Perkin Elmer) plate reader. Luciferase expression in RAW-Neg was used to normalize for changes to the minimal promoter that are not dependent on the enhancer elements.

Overview of TICs selected for this study. The experiments performed in this study examined the effects of 14 TICs, which are listed along with their chemical characteristics in Table 1. Overall, our objective was to examine a set of representative TICs that encompassed a variety of chemical and physical characteristics. The individual TICs selected for screening in this analysis were chosen based several factors. First, we sought to include hazardous chemicals with properties broadly representative of categories of TICs such as chlorides and cyanates that might pose a risk to human health if they were released intentionally in a terrorist incident. For practical and laboratory safety reasons, we limited our experimental analysis to low toxicity TICs in order to protect personnel against accidental exposure via the inhalation and/or dermal absorption pathways. Second, it was important to consider differences in physical properties, especially solubility to perform the experiments using cell cultures. TICs representing a range of solubilities were selected in order to accommodate differences in how the compounds might dissolve in various types of tissue culture media used in these experiments. Third, we selected several compounds that differed only by a single carbon, chirality, or in branching, in hopes of determining the extent to which subtle changes in structures could affect the outcome of interactions with macrophages.

This allows us to delineate subtle differentials in chemical properties that may modulate innate immune cell response as a function of chemical exposures. Using these criteria, our set of candidate TICs for screening included: $CH_2=CHCH_2NCS$, $AsCl_3$, $ClCOOCH_2CH_2CH_2CH_3$, CCl_4, $ClCH_2COCl$, $ClCOSC_2H_5$, $C_2H_5P(O)Cl_2$, HI, $ClCO_2CH_2CH(CH_3)_2$, $(CH_3)_2CHOCOCl$, $CH_2(C_6H_4NCO)_2$, CCl_3SCl, and $ClCO_2CH_2CH_2CH_3$. The abbreviations for each of these chemicals is included in Table 1, and such abbreviations will be referred in the following sections.

Optimizing TICs doses. Because our interests were in determining the effects of TICs at subcytolytic doses, in the first series of experiments we performed a dose-curve analysis to identify optimal sub-lethal concentrations of each TIC. In these experiments, RAW 264.7 mouse macrophages were plated in wells of a 96-well tissue culture plate and exposed to the indicated concentrations of TICs (Table 1). We chose to define amounts of TICs using 0.1% DMSO as a vehicle in different molar concentrations. The adoption of this experimental protocol was necessary because it is not possible to determine the exact solubility of these compounds in the complex medium necessary for growth of the macrophages. As shown in Figure 1, at 4 h following treatment only AITC caused greater than 50% cytotoxicity was detected in the population of treated macrophages. Several TICs caused greater than 25% cytotoxicity at one of the dilutions. Somewhat unexpectedly, treatment with the higher dilutions of four TICs (IsoButClF, IsoPropClF, 4'4 MetBis, Parat) resulted in more cytotoxicity than when the cells were exposed to the apparently higher concentration of this chemical. This non-linear association between those four TIC

concentrations and cytotoxicity may be due to concentration-dependent precipitations of these chemicals. This set of initial experiments identified sublytic dilutions of TICs and established optimal doses of each chemical to be used in the next series of experiments.

Chemokine production in TIC-exposed macrophages. In the next set of experiments we examined the effects of TICs on chemokine production in macrophages. Chemokines are released by macrophages in response to stimuli, and form a gradient that can be sensed by other leukocytes and then followed to the point of infection. Hence, the production and release of chemokines by macrophages supports a coordinated inflammatory response involving many cells of the host immune system. Moreover, chemokine production is hallmark of macrophages responding to a particular stimuli. For these reasons we chose to use chemokine production as one quantifiable method to follow the modulation of macrophage activity by TICs. In order to examine the production of multiple chemokines in a single sample we adopted a multiplex approach using individually tagged fluorescent beads that contained immunoreactive antibodies to each chemokine under investigation. Following reaction with the supernatants of the treated cells, the beads were sorted as described in the methods and the reactive intensity was determined for each particular chemokine. As a positive control we treated macrophages with LPS, a bacterial cell wall component known to stimulate macrophage production of chemokines. As a negative control macrophages were treated with culture medium alone. In all, each TIC was examined for the capacity to

trigger production of 5 chemokines, including MCP-1, MIP-1α, MIP-1β, MIP-2, and RANTES. Those TICs causing changes in chemokine levels are summarized in the panels of Figure 2. Of the chemokines tested only RANTES was not affected by any of the TICs tested. Six of the TICs triggered changes in either MCP-1, MIP-1α, MIP-1β, or MIP-2. Notably, AITC and Ars(III)Cl dramatically reduced levels of MCP-1, MIP-1α, and MIP-1β (see Figures 2B and 2C). ButClF and CCl_4 caused a marginal reduction in MCP-1 (see Figures 2D and 2E). IsoButClF and PropClF were the two TICs tested that increased levels of chemokines, and raised the amounts of MIP-1α and MIP-1β in the treated macrophages (see Figure 2F and 2G). Finally, PClMM reduced levels of MCP-1, MIP-1α, and MIP-1β (see Figure 2H), but not the same extent as AITC and Ars(III)Cl. An overall summary of the chemokines altered by each TIC is included in Table 2. Collectively, these data suggested that chemokine production can be altered by noncytolytic doses of TICs.

Cytokine production in TIC-exposed macrophages. In the next series of experiments we determined the impact of TICs on cytokine production in macrophages. Cytokines are molecules released by macrophages and modulate the activity of other immune cells in the body in an effort to tightly regulate immune responses. Thus, analysis of TIC-induced cytokine production in macrophages can reveal the extent of responsiveness of these cells to toxic chemicals. Using an experimental approach similar to that of the chemokine analysis, we examined the impact of each TIC on the production of selected cytokines. Again, LPS was used as a positive control in these experiments and treatment with culture medium alone was

included as the negative control. In all, we examined the expression of G-CSF, GM-CSF, IL-1α, IL-1β, IL-6, IL-10, IL-12p70, TNF-α and IFNγ in the presence of each TIC. Only those TICs causing a change in cytokine levels are summarized in Figure 3. In all 6 of the 14 TICs examined altered cytokine expression profiles with only, IL-1α, IL-1β, and TNF-α impacted by these chemicals. Similar to the chemokine results, AITC and Ars(III)Cl altered the expression of cytokines in macrophages. Interestingly, two cytokines IL-1α and IL-1β were increased in levels following exposure to these TICs, while TNF-α was decreased in the presence of these compounds, suggesting a more complex cytokine pattern (see Figures 3B and 3C). ButClF increased levels of IL-1α but had no other detectable impact on other cytokines examined (see Figure 3D). EtClTF and $EtPhCl_2$, which had no impact on chemokine expression, reduced levels of TNF-α (see Figure 3E and 3F). Finally, PClMM, which altered chemokine expression, showed a more subtle effect than AITC and Ars(III)Cl, but also increased levels of IL-1α and decreased levels of TNF-α (see Figure 3G). A summary of the impact of each TIC on cytokine production is provided in Table 2. Overall these findings indicate that specific TICs can alter the cytokine expression profiles of a subset of cytokines.

The Impact of TICs on Intracellular Signaling Pathway. In the next series of experiments we sought to determine which intracellular signaling pathways were modulated by particular TICs in cultured macrophages. Previous studies have shown that increased expression of chemokines and cytokines is directly regulated by activation of intracellular signaling pathways, which relay the extracellular

sensory information from the outside the cell and into the cytoplasm of the cell (2). Thus, it is plausible to assume that changes in chemokine and cytokine production should correspond to changes in intracellular signaling pathways. To address this possibility we chose to examine the ERK1/2, STAT3, JNK, p70 S6 kinase, IκB-α, STAT5A/B, CREB, and p38 pathways. Again, using the multi-plex approach we examined the changes in signaling patterns for each of these pathways. A hallmark of activation of these pathways is changes in the phosphorylation state of a central protein in a series of proteins that relay the signal in cascade fashion. Thus, increased phosphorylation was used as marker to identify activation of a signaling pathway.

Similar to the results examining cytokine and chemokine expression, not all of the TICs impacted signaling pathways in cultured macrophages. Those TICs having the most dramatic effects on signaling pathways are summarized in Figure 4. The signaling kinase JNK, was strongly activated by ButClF, CCl_4 and $EtPhCl_2$, and marginally activated by HA and PClMM (see Figure 4D-H). PClMM also activated p38 signaling, which was either unchanged or only slightly changed in the presence of other TICs (see Figure 4H).

As a second method to confirm the results from the multi-plex analysis, we performed immunoblot experiments using antibodies to phosphorylated p38. As shown in Figure 4I, when lysates from TIC-treated macrophages were examined for

increases in phosphorylated p38, we detected an increase in this protein, which corresponded to that observed in the multiplex analysis.

Unlike the results showing particular TICs repressing chemokine or cytokine production, we did not find any TIC that reduced the activation level of any particular signaling kinase. In all cases the changes involved an increase in the phosphorylation state of a certain kinase. A summary of the particular signaling pathways impacted by each TIC is provided in Table 2. As shown in Table 2, each of the TICs that caused changes in chemokine and cytokine levels also altered at least one signaling pathway.

Luciferase Reporter Data (Soumitra)

Discussion. In the current study we analyzed the impact of TICs on macrophage function in an effort to determine the mechanism by which these chemicals influence immune cell function, and perhaps damage the overall immune system. Results from these studies reveal several correlates between exposure to TICs and modulation of macrophage function, and support the idea of an uncoordinated immune response resulting from exposure to these chemicals. Indeed, the results indicate that both proinflammatory and anti-inflammatory events may take place in these cells after exposure to TICs. The data also shows that whether the macrophages become activated or repressed largely depends on the type of chemical used in the treatments. Moreover, the findings support the idea that changes in immune function could result from alterations in kinase signaling

pathways induced by the TICs. Collectively, these data provide the first comprehensive profile of the influence of low toxicity TICs on macrophage function.

Several of the TICs modulated the expression of chemokines in macrophages, with some TICs causing an increase in chemokines, while others suppressed chemokine production even below basal levels. For example, AITC, PCIMM, and As(III)Cl reduced levels of MCP-1, MIP-1α and MIP-1β, suggesting these TICs had a broad effect on chemokine production in macrophages. TICs such as CCl_4, ClACl, ETClTF, and ButClF, were more limited in their effects and only caused a marginal reduction in MCP-1, without any detectable influence on MIP-1α and MIP-1β. Only PropClF and IsoButClF triggered and increase in chemokine expression, but even then these changes were somewhat marginal and did not exceed more than two fold increases in MCP-1, MIP-1α, or MIP-1β. Finally, the remainder of the TICs examined in this study did not change the levels of chemokines to that different from the control.

We were curious as to whether there were any chemical similarities between chemokine-inducing or chemokine-repressing molecules. That is, was chemical structure similarity among the TICs used for this initial screening analysis associated with similar patterns in chemokine production? However, close examination of these chemical structures did not reveal any such pattern. In fact, the two chemicals, PropClF and IsoButClF, that induced chemokine production share the ClF group, but ButClF does was well and actually caused a decrease in chemokine production. Among the most significant inhibitors of chemokine

production - CCl_4, ClACl, ETCITF, and ButClF – there was no obvious shared chemical moiety, suggesting that physical characteristics such as solubility and stability could have an important influence on this process.

At the outset of these experiments we predicted that the TICs would have a pronounced impact on cytokine production. In our experience, even subtle changes in conditions can trigger cultured macrophages to secrete inflammatory cytokines. Yet, the changes we observed in cytokine production in the presence of TICs were marginal. In all we examined 7 different representative cytokines and only three of these (IL-1α, IL-1β, and TNFα) responded to the TICs under investigation in this study. That does not mean, however, that the cytokine responses demonstrated were not important. In fact, the opposite is likely to be the case. IL-1α, IL-1β, and TNFα are three crucial proinflammatory cytokines produced by macrophages, and changes in their expression alone could have an extensive effect on a coordinated immune response. Moreover, increased expression of the cytokines could lead to localized inflammatory damage. Similar to the chemokine results both As(III)Cl and AITC caused almost identical changes in the cytokine production profile.

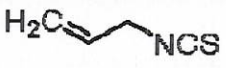
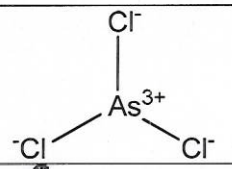
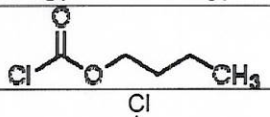
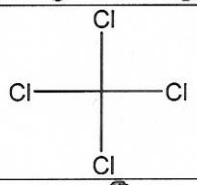
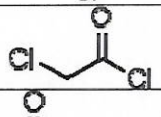
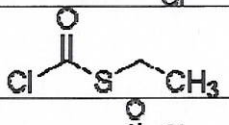
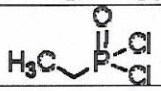
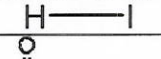
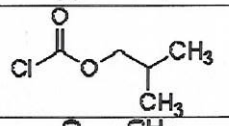
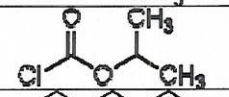
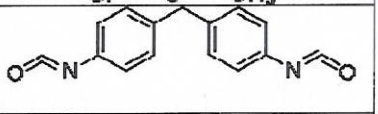
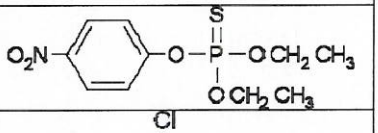
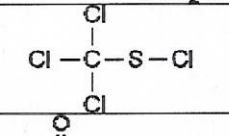
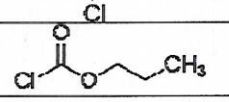
In an effort to begin to connect the TIC-induced changes in chemokine and cytokine production with modulation of intracellular signaling pathways, we examined the effects of each chemical on representative signaling pathways. Although determining the exact changes in signaling that lead to changes in cytokine and chemokine expression in TIC-treated macrophages will require further work, our

analysis indicates there are some important observations to consider. Most importantly, each TIC causing changes in cytokine and chemokine production also modulated at least one of the signaling pathways. The two TICs that had no effect on either chemokine or cytokine production also did not activate any of the signaling pathways. JNK was the signaling kinase most frequently modulated by the TICs with over half of the TICs examined causing an increase in activated levels of this kinase. It will be of particular interest in future studies to determine if signaling via JNK represents a general mechanism through which macrophages sense and respond to toxic molecules.

Collectively, our data indicate that exposure to low doses of TICs may cause severe disruption of the immune system, by disrupting the coordinated expression of select chemokines and cytokines. In some situations, which may largely depend on the type of TIC involved, this event may lead to localized tissue damage due to heightened inflammatory responses. In contrast, TICs attenuating responses may cause a variety of diseases that are prevented by effective immune surveillance.

Appendix Tables and Figures.

Table 1: Summary of TICs examined in this study

Chemical name	Molecular formula	Chemical structure
Allyl isothiocyanate (AITC)*	$CH_2=CHCH_2NCS$	
Arsenic(III) chloride (Ars(III)Cl) *	$AsCl_3$	
Butyl chloroformate (ButClF) *	$ClCOOCH_2CH_2CH_2CH_3$	
Carbon tetrachloride (CCl4) *	CCl_4	
Chloroacetyl chloride (ClACl) *	$ClCH_2COCl$	
S-Ethyl chlorothioformate (EtClTF) *	$ClCOSC_2H_5$	
Ethylphosphonic dichloride (EtPhCl2) *	$C_2H_5P(O)Cl_2$	
Hydriodic acid (HA) *	HI	
Isobutyl chloroformate (IsoButClF) *	$ClCO_2CH_2CH(CH_3)_2$	
Isopropyl chloroformate (IsoPropClF) *	$(CH_3)_2CHOCOCl$	
4,4′-Methylenebis (phenyl isocyanate) (4'4'MetBis) *	$CH_2(C_6H_4NCO)_2$	
Parathion solution (Parat) *		
Perchloromethyl mercaptan (PClMM) *	CCl_3SCl	
Propyl chloroformate (PropClF) *	$ClCO_2CH_2CH_2CH_3$	

*Abbreviation used in this report

Table 2: TICs' concentration used for cytokine/chemokine, signaling pathway & reporter induction.

	TICs	Stock conc. (M)	TCD_{50} (mM)	concentration used for induction (μM)
1	Allyl isothiocyanate	9.7	3.94	9.7
2	Arsenic(III) chloride	11.9	1.99	11.9
3	Butyl chloroformate	7.7	10.95	77
4	Carbon tetrachloride	10.3	11.81	103
5	Chloroacetyl chloride	12.3	1.75	123
6	Ethyl chlorothioformate	9.2	6.81	92
7	Ethylphosphonic dichloride	9.2	1.36	9.2
8	Hydriodic acid	7.6	8.1	76
9	Isobutyl chloroformate	7.6	11.5	76
10	Isopropyl chloroformate	1	1.14	10
11	4,4'-Methylenebis	7.992 mM	5.13 (μM)	80 nM
12	Parathion solution	0.34 mM	0.53 (μM)	3.4 nM
13	Perchloromethyl mercaptan	9.05	1.81	9.05
14	Propyl chloroformate	8.7	5.53	87

Table 3: summary of the TICs' cytokine/chemokine induction.

TICs	mIL-1a	mIL-1b	mIL-6	mTNFa	mG-CSF	mMCP-1	mMIP-1a	mMIP-1b	mMIP-2	mRANTES
AITC	–	++	+++	++	+++	–	—	—	++	–
Ars(III)Cl	++	++	+	+++	NC	——	——	——	+++	—
ButClF	—	++	—	++	+	NC	NC	–	NC	–
CCl_4	NC	++	NC	+	+	+	NC	NC	NC	+
ClACl	NC	+	—	+	+	–	–	–	NC	–
EtClTF	+	+++	NC	NC	+	++	NC	NC	NC	+
$EtPhCl_2$	++	++	NC	+	++	+	+	NC	NC	NC
HA	NC	++	NC	NC	+	+	NC	+	NC	NC
IsoButClF	+	–	++	+	+	+	–	++	NC	+
IsoPropClF	–	++	–	+	++	+	+	+	NC	NC
4'4'MetBis	NC	++	NC	+	++	+	+	+	+	NC
Parat	++	+	NC	NC	+	+	NC	NC	NC	–
PClMM	+	+	+	++	++	+	NC	NC	+	NC
PropClF	—	+	NC	+	+	+	NC	NC	+	NC

(+): induced; (-): suppressed, NC: no change

Table 4: summary of the TICs' signaling pathway induction.

TICs	CREB	Erk-MAP kinase 1/2	IkB-a	JNK	p38	p70 S6 kinase	STAT3	STAT5A/B
AITC	++	++	+	+	+	NC	++	+
Ars(III)Cl	++	+	++	++	+	+	++	+
ButClF	+	+	NC	NC	+	NC	+	NC
CCl_4	-	NC	--	--	--	--	-	--
ClACl	+	++	+	+	+	+	+	++
EtClTF	++	+	+	+	+	+	NC	++
$EtPhCl_2$	+	+	+	NC	+	+	NC	+
HA	+	+	+	+	+	+	+	+
IsoButClF	++	+	+	+	+	+	+	++
IsoPropClF	+	+	+	+	+	+	+	+
4'4'MetBis	+	+	+	+	+	+	+	+
Parat	+	+	+	+	+	+	+	+
PClMM	+	+	+	+	+	+	+	+
PropClF	+	NC	-	-	-	-	+	-

(+): induced; (-): suppressed, NC: no change

Table 5: summary of the TICs' reporter activity.

TICs	AP1	C/EBP	CRE	GAS	ISRE	NFAT	NFkB	RBP/Jk	SMAD	SRE	STAT3	TCF
AITC	+	-	+++	NC	NC	--	--	-	-	NC	-	-
Ars(III)Cl	---	+	NC	NC	NC	-	NC	NC	+	++	NC	+
ButClF	NC	NC	-	NC	NC	NC	NC	-	NC	+	NC	-
CCl_4	NC	NC	NC	NC	+	NC	+	NC	NC	+	NC	NC
ClACl	+++	++	++	NC	NC	+++	++	++	++	++	-	++
EtClTF	NC	NC	NC	NC	NC	+	NC	NC	++	+++	NC	NC
$EtPhCl_2$	+++	+++	++	+	NC	++	+++	NC	++	+	+	++
HA	++	++	++	+++	++	+++	+	+++	+++	+++	+	+++
IsoButClF	++	NC	NC	NC	NC	NC	NC	NC	+++	+++	+	NC
IsoPropClF	++	+	+	++	++	+	++	+	++	+	+	++
4'4'MetBis	NC	NC	-	NC	NC	-	NC	-	+	+++	NC	NC
Parat	NC	NC	NC	+	NC	NC	+	NC	NC	+	+	NC
PClMM	+	-	-	NC	NC	-	NC	--	NC	+++	-	-
PropClF	+	+	NC	+	NC	+	NC	+	+	NC	+	+

(+): induced; (-): suppressed, NC: no change

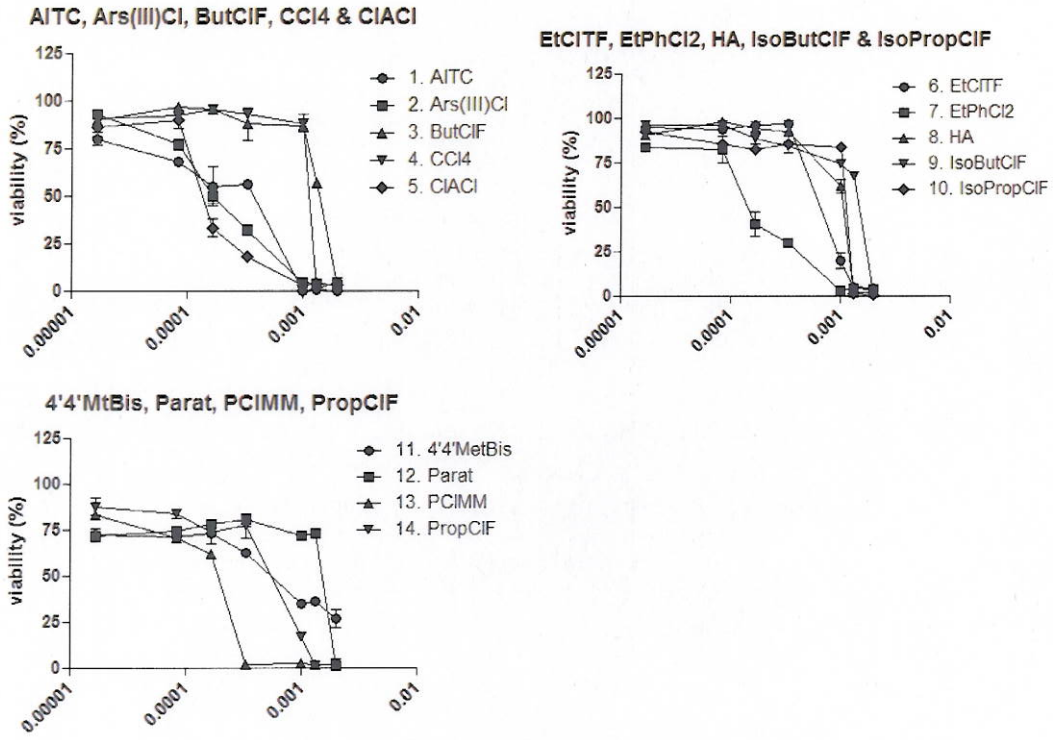
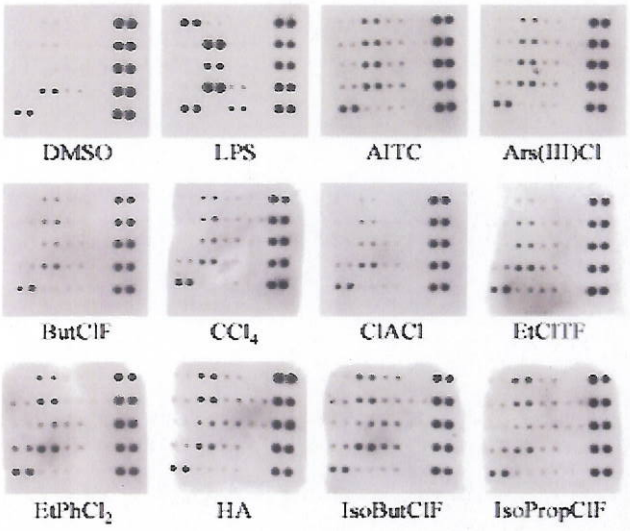


Figure 1. Cytotoxicity Dose Profiles of TICs. In order to determine the optimal doses of TICs to use in this study, cultured RAW 264.7 macrophages were incubated with a range of dilutions of each of these chemical. After 12 h of incubation the supernatants from the cells treated with these chemicals were examined for release of lactate dehydrogenase, an enzyme that is retained in the cytoplasm and only released from cells that have died. The results in panels A-C indicate the level of cytotoxicity and the corresponding dilution of each compound tested under these conditions. Specific methodology is included in the materials and methods. The results represent the mean of each treatment in triplicate and the error bars indicate the standard error calculated for each group.



A	G-CSF	IFN-γ	IL-1-α	IL-10	pos
B	M-CSF	TNF-α	IL-2	IL-12	pos
C	GM-CSF	IP-10	IL-4	IL-13	pos
D	MIG	Rantes	IL-5	neg	pos
E	MIP-1-α	VEGF	IL-6	neg	pos

Figure 2. Chemokine Expression Profiles in TIC-treated Macrophages. Using the standardized dose of each TIC, as summarized in Figure 1, RAW 264.7 macrophages were treated with each compound for 5 h and the supernatants were examined by multi-plex technology to determine changes in levels of the indicated chemokines. Only those TICs that altered chemokine production in macrophages are included in the figure. RANTES expression was not altered by any of the TICs tested, and only those chemokines changed by TICs are included in the panels. Panel A: Impact of LPS on chemokine production (positive control). Panels B-G: Impact of indicated TICs on chemokine levels. The name of the corresponding TIC is included in each panel. Experimental samples are shaded in grey and control (untreated) are indicated by the open bars. The data represents the mean of three experimental samples, and the error bars indicate the standard error of the mean.

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